

FORM PTO-1390 (Modified) (REV 11-98)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 00:117	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5) 09/673937	
INTERNATIONAL APPLICATION NO. PCT/JP 99/02205		INTERNATIONAL FILING DATE 23 April 1999		PRIORITY DATE CLAIMED 24 April 1998	
TITLE OF INVENTION Method Of Stabilizing Enzyme And Enzyme Composition					
APPLICANT(S) FOR DO/EO/US BABA, Toshiyuki TABATA, Hiromasa NAGAMATSU, Katashi WATAZU, Yoshifumi					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
<ol style="list-style-type: none"> 1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1). 4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371 (c) (2)) <ol style="list-style-type: none"> a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). 7. <input checked="" type="checkbox"/> A copy of the International Search Report (PCT/ISA/210). 8. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3)) <ol style="list-style-type: none"> a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 9. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 10. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). 11. <input checked="" type="checkbox"/> A copy of the International Preliminary Examination Report (PCT/IPEA/409). 12. <input checked="" type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)). 					
Items 13 to 20 below concern document(s) or information included:					
<ol style="list-style-type: none"> 13. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 14. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 15. <input type="checkbox"/> A FIRST preliminary amendment. 16. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 17. <input type="checkbox"/> A substitute specification. 18. <input type="checkbox"/> A change of power of attorney and/or address letter. 19. <input type="checkbox"/> Certificate of Mailing by Express Mail 20. <input checked="" type="checkbox"/> Other items or information: <div style="border: 1px solid black; padding: 5px; margin-top: 5px;"> Transmittal Sheets in duplicate w/fees charged to Dep. Acct. 07-2100 Translation of Japanese Text Application (36 pages) No Drawings Executed Declaration Assignment to International Reagents Corporation Copy of PCT/RO/101, PCT/ISA/210/220 Copy of PCT/RO/105, PCT/IB/301, PCT/IB/304 Copy of PCT/IPEA/416, 409, 401 </div> 					

APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5) 09/673937		INTERNATIONAL APPLICATION NO. PCT/JP 99/02205		ATTORNEY'S DOCKET NUMBER 00:117	
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21. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :				CALCULATIONS PTO USE ONLY	
<input type="checkbox"/>	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	\$1,000.00			
<input checked="" type="checkbox"/>	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	\$860.00			
<input type="checkbox"/>	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	\$710.00			
<input type="checkbox"/>	International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)	\$690.00			
<input type="checkbox"/>	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)	\$100.00			
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).				\$0.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	9 - 20 =	0	x \$18.00	\$0.00	
Independent claims	- 3 =	0	x \$80.00	\$0.00	
Multiple Dependent Claims (check if applicable).			<input type="checkbox"/>	\$0.00	
TOTAL OF ABOVE CALCULATIONS =				\$860.00	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable).			<input type="checkbox"/>	\$0.00	
SUBTOTAL =				\$860.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).			+	\$0.00	
TOTAL NATIONAL FEE =				\$860.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).			<input checked="" type="checkbox"/>	\$40.00	
TOTAL FEES ENCLOSED =				\$900.00	
				Amount to be: refunded	\$
				charged	\$

☐ A check in the amount of _____ to cover the above fees is enclosed.

☒ Please charge my Deposit Account No. **07-2100** in the amount of **\$860.00** to cover the above fees.
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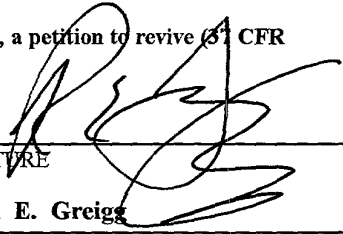
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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NAME

31,517

REGISTRATION NUMBER

24 October 2000

DATE

DESCRIPTION

METHOD OF STABILIZING ENZYME AND ENZYME COMPOSITION

Technical Field

The present invention relates to a method of stabilizing an enzyme contained in a medium and an enzyme composition, with regard to a control substance. The control substance is used to fulfill the following roles, that is, those containing a certain amount of an enzyme as a component are used as the control substance and applied to an analyzer in an test for measuring the amount of the enzyme contained in human blood, whereby the analyzer inspects whether or not an accurate value of the enzyme can be detected. Alternatively, the numerical value of measurement of the control substance is previously obtained, whereby an accurate amount of the enzyme is obtained from the measurement value obtained in an actual specimen by proportioning.

Background Art

Aspartate aminotransferase EC 2.6.1.1 (hereinafter abbreviated to AST) is an enzyme capable of producing glutamic acid and oxaloacetic acid from aspartic acid and α -ketoglutaric acid and mainly exists in heart, liver and skeletal muscle, and is a useful index for diagnosis of acute hepatitis, chronic hepatitis and cardiac infarction.

Alanine aminotransferase EC 2.6.1.2 (hereinafter abbreviated to ALT) is an enzyme capable of producing glutamic acid and pyruvic acid from alanine and α -ketoglutaric acid and mainly exists in liver, kidney and heart, and is a useful index for clinical diagnosis similar to AST.

In an ordinary test performed to measure the amount of AST and ALT contained in blood, the control substance is often used.

Accordingly, handling of the control substance has become important to secure the stability and reliability of an test value in an ordinary test and to perform a clinical trial to which an accurate and high-level technique is required. For example, in case the measurement value of AST or ALT contained in human blood is detected, a control substance containing AST or ALT is used. However, it is necessary to sufficiently stabilize the enzyme activity of AST or ALT as an unstable enzyme incorporated in the control substance to sufficiently fulfill the roles of the control substance described above.

From such a point of view, the prior arts with the object of stabilizing the enzyme incorporated in the control substance are disclosed in Unexamined Patent Publication (Kokai) No. Sho 55-141194, Unexamined Patent Publication (Kokai) No. Sho 56-148291 and Unexamined Patent Publication (Kokai) No. Sho 57-45453. In these prior arts, ethylene

glycol, sucrose or glycerol is used as the stabilizer.

The stabilization of the enzyme using amino acid has hitherto been studied by Harold L. Segal et. al. (Biochemical and Biophysical Research Community cations, Vol. 30 (1), pages 63-68, 1968).

Although there is also a report using amino acid to reduce the turbidity of the control substances, it is considered that amino acid can prevent denaturation of protein in either case.

However, a conventional stabilizer such as ethylene glycol, sucrose or glycerol must be used in high concentration so that the stabilizer exhibits the effect thereof. That is, the concentration of ethylene glycol and that of glycerin must be became higher, for example, about 5 mol/L and 3.3 mol/L, while sucrose must be added in the proportion within a range from 1 to 10%. Therefore, the control substance itself has high specific gravity and/or high viscosity to cause such a problem that the control substance, which should serve as a parameter, has physical properties different from those of human serum. Such a problem leads to the state where an intrinsic object of the control substance itself can not be attained, for example, it produces a factor for causing a difference in measured value between types of a latest automatic analyzer and equipments when applying the control substance to the analyzer because the accuracy of sampling

is different from that of common human serum (Japan Clinical Chemistry Society, Scientific Liaison Committee, Clinical Chemistry Vol. 25 (2), pages 135-148, 1996).

Disclosure of the Invention

To solve the problems described above, the present inventors have studied intensively based on the fact such as roles of amino acid to be fulfilled to the turbidity of the control substances and denaturation of protein. As a result, they have found that valine and proline particularly stabilize the enzyme such as AST or ALT contained in the control substance, thus completing the method of stabilizing an enzyme and enzyme composition of the present invention.

The first feature of the method of stabilizing an enzyme according to the present invention is to incorporate, as a stabilizer for stabilizing at least one enzyme selected from the group consisting of aspartate aminotransferase and alanine aminotransferase, at least one amino acid selected from valine and proline in at least one medium selected from the group consisting of serum and buffer. With a proviso that said proline is incorporated in the amount which exceeds 100 mmol/L when using said proline alone as said stabilizer in case an object to be stabilized is alanine aminotransferase and aspartate aminotransferase is not contained.

The second feature of the method of stabilizing an

enzyme according to the present invention is to control the content within a range from 0.5 to 100 mmol/L when using valine alone among valine and proline, in addition to the first feature.

The third feature of the method of stabilizing an enzyme according to the present invention is to control the valine content within a range from 5 to 20 mmol/L and to control the proline content within a range from 10 to 500 mmol/L when using valine and proline in combination, in addition to the first feature.

The fourth feature of the method of stabilizing an enzyme according to the present invention is to control the content within a range from 0.5 to 500 mmol/L when using proline alone among valine and proline for stabilization of aspartate aminotransferase, in addition to the first feature.

The fifth feature of the method of stabilizing an enzyme according to the present invention is that said serum or buffer is a buffer containing a soluble protein, in addition to the first feature.

The six feature of the method of stabilizing an enzyme according to the present invention is that said soluble protein is at least one soluble protein selected from the group consisting of albumin and gelatin, in addition to the fifth feature.

The seventh feature of the method of stabilizing an

enzyme according to the present invention is that the concentration of said albumin is within a range from 0.5 to 15W/V%, in addition to the sixth feature.

The eighth feature of the method of stabilizing an enzyme according to the present invention is that the concentration of said gelatin is within a range from 0.5 to 15W/V%, in addition to the sixth feature.

The feature of the enzyme composition is to comprise at least one medium selected from the group consisting of serum and buffer, at least one enzyme selected from the group consisting of aspartate aminotransferase and alanine aminotransferase and a stabilizer for stabilizing said enzyme, which is made of at least one amino acid selected from valine and proline, said enzyme and said stabilizer being incorporated in said medium. With a proviso that said proline is incorporated in the amount which exceeds 100 mmol/L when using said proline alone as said stabilizer in case an object to be stabilized is alanine aminotransferase and aspartate aminotransferase is not contained.

In the above description, the medium refers to a mother liquor such as solvent or dispersion medium in which an enzyme or a stabilizer is dissolved or dispersed, and those prepared by dissolving or dispersing the enzyme and stabilizer in the medium can be used as the control substance. In case the object to be test is contained in human serum, human serum

or a similar one prepared by treating the human serum is preferably used as the medium for control substance. That is, it is preferable to select, as the medium, those having properties similar to those of the object to be examined.

As the medium, for example, serum and buffer can be used. The serum refers to human serum, serum of other animals, or treated serums in a broad sense. The serum and buffer may be a buffer containing a soluble protein solution. Hereinafter, aspartate aminotransferase is abbreviated to AST, while alanine aminotransferase is abbreviated to as ALT.

Valine and proline as the stabilizer for stabilizing AST and ALT are used alone or in combination thereof. As a matter of course, these stabilizers are used to stabilize AST and ALT alone or in combination, thereby to exert good effect. However, the case of using as the stabilizer to other enzymes is also included in the scope of the present invention. When using proline alone to stabilize ALT, the amount of proline preferably exceeds 100 mmol/L.

In the features described above, those containing AST as the enzyme in the medium can be used as the control substance, typically, in case the amount of AST contained in human blood is measured. Those containing ALT as the enzyme in the medium can be used as the control substance, typically, in case the amount of ALT contained in human blood is measured.

The origin of AST and ALT to be incorporated in the

control substance includes, but is not specifically limited to, biological substance such as bovine heart, pig heart, human heart, serum, red blood cell and urine, those prepared by cultivating human cells or those prepared by cultivating a transforming cell integrated with a human-derived gene. In this case, the content of AST and that of ALT are respectively controlled within a range from 5 to 1000 U/L, and preferably from 30 to 500 U/L.

In the features described above, when the medium is serum, the control substance contains an enzyme and amino acid as the stabilizer in serum.

In the features described above, when the medium is buffer, for example, a BES buffer prepared by dissolving bovine serum albumin can be used. However, those prepared by dissolving various substances in buffer can be used to have chemically or physically similar properties corresponding to types and states of the object to be examined (strictly a medium of the object to be examined) to be subjected actually to an examining apparatus. The present invention also includes such a case.

The buffer used in the present invention includes, for example, organic amine and Good's buffers whose pH can be adjusted appropriately within a range from 6.0 to 8.5; and biochemical buffers such as citric acid-sodium diphosphate buffer, hydrochloric acid-sodium veronal-sodium acetate

buffer, potassium monophosphate-sodium diphosphate buffer, potassium monophosphate-borate buffer, potassium monophosphate-sodium hydroxide buffer, hydrochloric acid-collidine buffer, hydrochloric acid-sodium veronal buffer, hydrochloric acid-tris(hydroxymethyl)aminomethane buffer, hydrochloric acid-borate buffer, boric acid-sodium carbonate buffer, boric acid-borate buffer, hydrochloric acid-aminomethylpropanediol buffer, ammonium chloride-ammonia buffer, glycine-sodium hydroxide buffer, boric acid-sodium hydroxide buffer, hydrochloric acid-sodium dimethylglycine buffer, borate-sodium hydroxide buffer, borate-sodium carbonate buffer, Sørensen buffer, glycine-sodium chloride-hydrochloric acid buffer, sodium dicitrate-hydrochloric acid buffer, sodium diacetate-sodium hydroxide buffer, borate-sodium hydrochloride buffer, Michaelis buffer, sodium veronal-sodium acetate-hydrochloric acid buffer, Clark-Lub's buffer, boric acid-potassium hydrochloride-sodium hydroxide buffer, Atkins-Pantin buffer, Palitzsch buffer, Kolthoff buffer, McIlvaine buffer, Hasting-Sendroy buffer, Britton-Robinson buffer, maleate buffer, tris-maleate buffer, veronal buffer and veronal-acetate buffer. Buffers other than these buffers can also be used as far as they have buffering capacity at the pH within a range from 6.0 to 8.5.

The organic amine buffer includes, for example, diethanolamine buffer, 2-ethylaminoethanol buffer, 2-

amino-2-methyl-1-propanol and N-methyl-D-glucamine.

The Good's buffer includes, for example, MES (2-(N-Morpholino)ethanesulfonic acid) buffer, Bis-Tris (Bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane) buffer, ADA (N-(2-Acetamido)iminodiacetic acid) buffer, PIPES (Piperazine-N,N'-bis(2-ethanesulfonic acid) buffer, ACES (N-(2-Acetamido)-2-aminoethanesulfonic acid) buffer, MOPSO (3-(N-Morpholino)-2-hydroxypropanesulfonic acid) buffer, BES (N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid) buffer, MOPS (3-(N-Morpholino)propanesulfonic acid) buffer, TES (N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) buffer, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, DIPSO (3-[N,N-Bis(2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid) buffer, TAPSO (N-Tris(hydroxymethyl)methyl-2-hydroxy-3-aminopropanesulfonic acid) buffer, POPSO (Piperazine-N,N'-bis(2-hydroxypropanesulfonic acid) buffer, HEPPSO (N-2-hydroxyethylpiperazine-N-2-hydroxypropane-3-sulfonic acid) buffer, EPPS (N-2-Hydroxyethylpiperazine-N'-3-propanesulfonic acid, another name: HEPPS) buffer, Tricine (Tris(hydroxymethyl)methylglycine) buffer, Bicine (N,N-bis(2-hydroxyethyl)glycine) buffer, TAPS (N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid) buffer, CHES (2-(Cyclohexylamino)ethanesulfonic acid) buffer, CAPSO (3-N-Cyclohexylamino-2-hydroxypropanesulfonic

acid) buffer and CAPS (3-Cyclohexylaminopropanesulfonic acid) buffer.

When using the organic amine buffer, it may be used as an aqueous medium whose concentration was adjusted within a range from 20 mM to 2 M, preferably from 20 mM to 1 M, and most preferably from 20 mM to 500 mM. Preferable aqueous medium is specifically purified water, and coenzymes, soluble salts, surfactants, stabilizers and antiseptics may also be appropriately incorporated.

When using the Good's buffer or biochemical buffer, it may be used after adjusting the concentration within a range from 20 mM to 1 M, preferably from 20 mM to 500 mM, and most preferably from 20 mM to 300 mM.

In the features described above, when the medium is a soluble protein solution, the soluble protein solution includes aqueous solutions of BSA, human serum albumin (HSA) and gelatin and these aqueous solutions can be used alone or in combination. In this case, the concentration of albumin and that of gelatin are respectively within a range from 0.5 to 15W/V%. When the medium is serum, the buffers described above can also be appropriately used.

In case the enzyme is stabilized by using valine or proline, valine and proline are not added in a large amount so that the resulting control substance has neither high specific gravity, nor high viscosity. Furthermore, valine

and proline did not exert a negative influence on stabilization of enzymes other than AST and ALT, for example, alkaline phosphatase (ALP) (ES. 3.1.3.1), creatine kinase (CK) (EC. 2.7.3.2), lactate dehydrogenase (LDH) (EC. 1.1.1.27) and γ -glutamyl transferase (γ -GTP) (EC. 2.3.2.2). With regard to the amount of each enzyme to be added, for example, the amount of ALP is preferably within a range from 9 to 6500 U/L, particularly preferably from 45 to 1300 U/L; the amount of CK is preferably within a range from 6 to 4000 U/L, particularly preferably from 30 to 800 U/L; the amount of LDH is preferably within a range from 8 to 4000 U/L, particularly preferably from 40 to 800 U/L; and the amount of γ -GTP is preferably within a range from 2 to 1200 U/L, particularly preferably from 10 to 800 U/L.

When using valine or proline as the stabilizer of the control substance containing at least one enzyme selected from the group consisting of AST and ALT, the content of valine used alone is controlled within a range from 0.5 to 100 mmol/L. More preferably, the content of valine is controlled within a range from 10 to 20 mmol/L. It is made possible to exert good stabilization effect and to enable physical and chemical properties to bear resemblance to those of serum as the object to be examined by controlling each content within the above range.

The content of proline used alone is controlled within

a range from 0.5 to 500 mmol/L. More preferably, the content of proline is controlled within a range from 100 to 500 mmol/L. When using proline alone to stabilize ALT (in case of containing no AST), proline is preferably incorporated in the amount which exceeds 100 mmol/L, more preferably within a range from 200 to 500 mmol/L, and most preferably from 300 to 500 mmol/L.

With regard to the content when using valine and proline in combination, for example, the content of valine is controlled within a range from 5 to 20 mmol/L, the content of proline is controlled within a range from 10 to 500 mmol/L, and the total content of them used in combination is controlled within a range from 15 to 520 mmol/L.

It is made possible to exert good stabilization effect and to enable physical and chemical properties to bear resemblance to those of serum as the object to be examined by controlling each content within the above range. Thus, it is made possible to continually make physical and chemical properties of the control substance resemble to those of serum as the object to be examined, secure the stability, obtain the reliability of the resulting data, and eliminates a difference between equipments where the examination is performed.

According to the method of stabilizing an enzyme based on the feature of the present invention, it is made possible

to inhibit denaturation of AST or ALT or a combination thereof in a medium thereby to stabilize them by incorporating valine and proline alone or in combination.

It is also made possible to stabilize AST or ALT by the stabilizer and to obtain stable and accurate detected data in the examination whose object to be examined is AST or ALT. By using valine or proline or a combination thereof as the stabilizer to AST or ALT, particularly, these enzymes can be sufficiently stabilized by a small content of valine or proline.

According to the method of stabilizing an enzyme based on the feature of the present invention, it is made possible to use the control substance wherein AST or ALT as well as valine or proline as the stabilizer thereof are added in serum as the medium and to detect under good environment which is physically and chemically similar when AST or ALT contained in serum is detected by using the medium as serum.

Similarly, AST or ALT can be stabilized with valine or proline as the stabilizer in a stable medium such as buffer by using the medium as the buffer. As a matter of course, a control substance having physical and chemical properties similar to those of the object to be examined (solvent) by optionally dissolving various components in the buffer.

In the method of stabilizing an enzyme based on the feature of the present invention, when using valine alone

among valine and proline, AST or ALT can be satisfactorily stabilized by controlling the content within a range from 0.5 to 100 mmol/L.

In the method of stabilizing an enzyme based on the feature of the present invention, when using valine and proline in combination, AST or ALT can be satisfactorily stabilized by controlling the valine content within a range from 5 to 20 mmol/L and controlling the proline content within a range from 10 to 500 mmol/L.

In the method of stabilizing an enzyme based on the feature of the present invention, when using proline alone among valine and proline, AST or ALT can be satisfactorily stabilized by controlling the content within a range from 0.5 to 500 mmol/L.

In the method of stabilizing an enzyme based on the feature of the present invention, in case the serum or buffer is buffer containing a soluble protein, AST or ALT as the stabilizer can be satisfactorily stabilized by valine or proline.

In case the soluble protein is at least one soluble protein selected from the group consisting of albumin and gelatin, AST or ALT can be stabilized by valine or proline as the stabilizer in a buffer containing albumin or gelatin. In this case, AST and ALT can be preferably stabilized when the concentration of albumin and that of gelatin are

respectively within a range from 0.5 to 15W/V%.

In the method of stabilizing an enzyme based on the feature of the present invention, in case at least one medium selected from the group consisting of serum and buffer contains ALT and does not contain AST, ALT can be preferably stabilized by incorporating proline in the amount which exceeds 100 mmol/L when using proline alone as the stabilizer for stabilizing ALT.

According to the enzyme composition of the present invention, there can be provided an enzyme composition, which is not liable to cause denaturation of AST and/or ALT and can exist in the stable state, by incorporating at least one enzyme selected from the group consisting of AST and ALT and the stabilizer for stabilizing the enzyme, which is made of at least one amino acid selected from valine and proline in at least one medium selected from the group consisting of serum and buffer. Therefore, it is made possible to obtain stable and accurate detected data by using such an enzyme composition, as the control substance, in the examination whose object to be examined is AST or ALT.

Best Mode for Carrying Out the Invention

Examples of the preparation of a control substance comprising valine or proline as a stabilizer and AST or ALT as an enzyme component according to the present invention will

now be described.

Using human serum (manufactured by TORINA CO. (Switzerland)), an endogenous enzyme is devitalized by previously subjecting to a heat treatment at 56°C for four hours and then sterilized by filtration using a membrane filter of 0.2 μ m in pore size (solution thus obtained is referred to as a human serum base). For example, 10 mmol/L of valine is dissolved in this human serum base (medium) and a certain amount of AST or ALT is dissolved furthermore, thereby making it possible to obtain a control substance containing valine as a stabilizer and AST or ALT.

For example, 300 mmol/L of proline is dissolved in the human serum base and a certain amount of AST or ALT is dissolved furthermore, thereby making it possible to obtain a control substance containing proline as a stabilizer and AST or ALT.

Similarly, examples of the preparation of a control substance comprising valine or proline as a stabilizer, AST or ALT as an enzyme component and a buffer as a medium according to the present invention will now be described.

Bovine serum albumin of 3W/V% (manufactured by INTERGEN CO. (U.S.A)) is incorporated in a BES buffer (20 mmol/L) and then sterilized by filtration (pH 7.4, solution thus obtained is referred to as a BSA base). For example, 10 mmol/L of valine is dissolved in this BSA base and a certain amount of AST or ALT is dissolved furthermore, thereby making it possible to

obtain a control substance containing valine as a stabilizer and AST or ALT in the buffer medium.

For example, 300 mmol/L of proline is dissolved in the BSA base and a certain amount of AST or ALT is dissolved furthermore, thereby making it possible to obtain a control substance containing proline as a stabilizer and AST or ALT in the buffer medium.

Furthermore, the control substance obtained by the method of the present invention is in the state of liquid on common use, but may be in the state other than liquid, such as freeze-dried product, cold-stored product and frozen liquid product.

Examples

The following examples further illustrate the present invention, but the present invention is not limited by the examples.

Example 1

Confirmation of stabilization effect of various amino acids on AST or ALT in control substance

Using a human serum base and a BSA base as a medium, control substances were prepared respectively by adding, as a stabilizer, no amino acid, valine (10 mmol/L) as amino acid, proline (10 mmol/L) or other amino acid (10 mmol/L, provided that 1 mmol/L in case of tyrosine) to the respective mediums

and then adding, as an enzyme, AST (about 100 U/L) or ALT (about 100 U/L).

The respective control substances thus obtained were stored at 45°C for four days and the residual activity of AST and ALT contained in the respective control substances was measured.

AST used herein is AST derived from a human liver gene recombinant (hereinafter referred to as r-AST) manufactured by ASAHI CHEMICAL INDUSTRIES CO., LTD. (manufacturing No. T-70). ALT used herein is ALT derived from a human liver gene recombinant (hereinafter referred to as r-ALT) manufactured by ASAHI CHEMICAL INDUSTRIES CO., LTD. (manufacturing No. T-71). Furthermore, the human serum base used herein is a human serum base prepared by previously subjecting human serum to a heat treatment at 56°C for four hours thereby to devitalize an endogenous enzyme, followed by sterilization by filtration using a membrane filter of 0.2 μ m in a pore diameter. The BSA base used herein is BES buffer (20 mmol/L) containing 3W/V% BSA.

With regard to the residual activity of AST and that of ALT, the residual activity of AST was measured by using an AST reagent L "KOKUSAI" manufactured by INTERNATIONAL REAGENTS CORPORATION. The residual activity of ALT was measured by using an ALT reagent L "KOKUSAI" manufactured by INTERNATIONAL REAGENTS CORPORATION.

[illegible]

Table 1

Stabilizer	Medium				
	BSA base		Human serum base		
Amino acid (10 mmol/L)	Residual activity (%) of AST	Residual activity (%) of ALT	Residual activity (%) of AST	Residual activity (%) of ALT	Residual activity (%) of ALT
No addition	53	32	41		20
Valine	85	62	71		55
Proline	73	61	54		35
Alanine	39	28	33		15
Leucine	46	28	40		20
Isoleucine	50	28	42		18
Methionine	48	31	40		20
Tryptophane	52	30	38		18
Phenylalanine	53	31	40		19
Glycine	53	30	40		19
Serine	52	32	40		19
Threonine	50	31	37		18
Cysteine	47	24	32		14
Tyrosine	49	31	40		18
Asparagine	41	25	37		14
Glutamine	43	15	32		17
Lysine	53	31	39		20
Histidine	47	28	36		15
Arginine	50	30	39		18
Aspartic acid	32	23	20		13
Glutamic acid	31	28	25		15

As is apparent from Table 1, valine and proline exerted preferable stabilization effect on AST and ALT.

Example 2

Confirmation of stabilization effect of valine on AST or ALT in control substance

Valine, r-AST and r-ALT were added to a human serum base and a BSA base in the concentration of about 100 U/L and, after storage at 45°C for four days, the residual activity was measured. The measurement of the activity of AST and ALT was performed in the same manner as in Example 1.

The results are shown in Table 2.

Table 2

Stabilizer	Medium				
	BSA base		Human serum base		
	Residual activity (%) of AST	Residual activity (%) of ALT	Residual activity (%) of AST	Residual activity (%) of ALT	Residual activity (%) of ALT
0	53	32	41	20	20
0.5	65	38	47	28	28
5	74	46	52	33	33
10	85	62	71	55	55
20	82	65	73	56	56
50	83	64	73	56	56
100	82	65	72	55	55

As is apparent from Table 2, the residual activity of AST and that of ALT were respectively 53% and 32% in case of no addition of valine in the BSA base, while the residual activity of AST and that of ALT became 65% and 38% respectively by adding 0.5 mmol/L of valine, thus improving the stability. The residual activity of AST and that of ALT were respectively 85% and 62% when the concentration of valine is 10 mmol/L, while the residual activity of AST and that of ALT were respectively 82% and 65% when the concentration of valine is 20 mmol/L. Furthermore, the residual activity of AST and that of ALT were respectively 82% and 65% when the concentration of valine is 100 mmol/L.

Similarly, the residual activity of AST and that of ALT were respectively 41% and 20% in case of no addition of valine in the human serum base, while the residual activity of AST and that of ALT became 47% and 28% respectively by adding 0.5 mmol/L of valine, thus improving the stability. The residual activity of AST and that of ALT were respectively 71% and 55% when the concentration of valine is 10 mmol/L, while the residual activity of AST and that of ALT were respectively 73% and 56% when the concentration of valine is 20 mmol/L. Furthermore, the residual activity of AST and that of ALT were respectively 72% and 55% when the concentration of valine is 100 mmol/L.

As is apparent from the above description, the

concentration of valine is preferably controlled within a range from 0.5 to 100 mmol/L, and more preferably from 10 to 20 mmol/L.

Example 3

Confirmation of stabilization effect of proline on AST or ALT in control substance

Using a human serum base and a BSA base as a medium, control substances were prepared respectively by adding, as a stabilizer, no proline and proline in the amount of 0, 5, 10, 100, 300 or 500 mmol/L to the respective mediums and then adding, as an enzyme, r-AST (about 100 U/L) or r-ALT (about 100 U/L). The respective control substances thus obtained were stored at 45°C for four days and the residual activity of AST and ALT contained in the respective control substances was measured. The results are shown in Table 3.

Table 3

Stabilizer	Medium				
	BSA base		Human serum base		
	Residual activity (%) of AST	Residual activity (%) of ALT	Residual activity (%) of AST	Residual activity (%) of ALT	Residual activity (%) of ALT
0	53	32	41	20	
0.5	60	41	49	34	
10	65	57	54	43	
100	74	69	67	65	
300	84	84	82	83	
500	84	85	85	88	

As is apparent from Table 3, the residual activity of AST and that of ALT were respectively 53% and 32% in case of no addition of proline in the BSA base, while the residual activity of AST and that of ALT became 60% and 41% respectively by adding 0.5 mmol/L of proline, thus improving the stability. The residual activity of AST and that of ALT became 74% and 69% respectively by adding 100 mmol/L of proline. Furthermore, the residual activity of AST and that of ALT were respectively 84% and 84% when the concentration of proline is 300 mmol/L, while the residual activity of AST and that of ALT were respectively 84% and 85% when the concentration of proline is 500 mmol/L.

Similarly, the residual activity of AST and that of ALT were respectively 41% and 20% in case of no addition of proline in the human serum base, while the residual activity of AST and that of ALT became 49% and 34% respectively by adding 0.5 mmol/L of proline, thus improving the stability. The residual activity of AST and that of ALT became 67% and 65% respectively by adding 100 mmol/L of proline, while the residual activity of AST and that of ALT became 82% and 83% respectively by adding 300 mmol/L of proline. Furthermore, the residual activity of AST and that of ALT were respectively 85% and 88% when the concentration of proline is 500 mmol/L.

As is apparent from the above description, the concentration of proline is preferably controlled within a

range from 0.5 to 500 mmol/L, and more preferably from 100 to 500 mmol/L. With regard to the concentration of proline to stabilization of ALT, it has been found that the concentration preferably exceeds 100 mmol/L and is less than 2.5 mol/L, and most preferably within a range from 300 to 500 mmol/L.

Example 4

Confirmation of stabilization effect of combination of valine and proline on AST or ALT in control substance

Valine, proline, r-AST and r-ALT were added to a human serum base in the concentration of about 100 U/L and, after storage at 45°C for four days, the residual activity was measured. The measurement of the activity of AST and ALT was performed in the same manner as in Example 1. The results are shown in Table 4.

Table 4

Stabilizer		Concentration of proline (mmol/L)	Medium		
Concentration of valine (mmol/L)			Human serum base	Residual activity (%) of ALT	Residual activity (%)
0	0	0	41		20
0	10	10	54		43
0	100	100	67		65
0	300	300	82		83
0	500	500	85		88
5	0	0	52		33
5	10	10	66		54
5	100	100	76		77
5	300	300	85		88
5	500	500	89		90
10	0	0	71		55
10	10	10	76		75
10	100	100	82		86
10	300	300	90		92
10	500	500	92		92
20	0	0	73		56
20	10	10	78		79
20	100	100	84		85
20	300	300	90		92
20	500	500	94		94

As is apparent from Table 4, the retention of AST was 41% in case of no addition of amino acid, while the retention of AST became 52% by adding 5 mmol/L of valine alone and the residual activity of AST became 54% by adding 10 mmol/L of proline alone and, furthermore, the retention of AST became 66% by adding 5 mmol/L of valine and 10 mmol/L of proline, thus improving the stability as compared with the case when using each amino acid alone.

The residual activity of AST was 20% in case of no addition of amino acid, while the residual activity of AST became 33% by adding 5 mmol/L of valine alone and the residual activity of AST became 43% by adding 10 mmol/L of proline alone and, furthermore, the residual activity of AST became 54% by adding 5 mmol/L of valine and 10 mmol/L of proline, thus improving the stability as compared with the case when using each amino acid alone.

The residual activity of AST was 78% and that of ALT was 79% when using 20 mmol/L of valine in combination with 10 mmol/L of proline, while the residual activity of AST was 84% and that of ALT was 85% when using 20 mmol/L of valine in combination with 100 mmol/L of proline, the residual activity of AST was 90% and that of ALT was 92% when using 20 mmol/L of valine in combination with 300 mmol/L of proline, and the retention of AST was 94% and that of ALT was 94% when using 20 mmol/L of valine in combination with 500 mmol/L of

proline.

As is apparent from the above description, when using valine in combination with proline, the concentration of valine is preferably controlled within a range from 5 to 20 mmol/L, the concentration of proline is preferably controlled within a range from 10 to 500 mmol/L, and the total concentration of valine and proline is preferably controlled within a range from 15 to 520 mmol/L. At the concentration described above, the specific gravity and viscosity bear resemblance to those of human serum.

Example 5

Confirmation of stabilization effect of valine or proline on AST or ALT in control substance

Control substances were prepared respectively by adding, as a stabilizer, 10 mmol/L of valine and 300 mmol/L of proline, no valine or no proline to a human serum base and then adding various AST(s) and ALT(s) derived from different origins in the concentration of about 100 U/L. The respective control substances thus obtained were stored at 45°C for four days and the residual activity was measured. In the same operation as in Example 1, the measurement of the activity of AST and ALT was performed. The results are shown in Table 5.

Table 5

Name of enzyme	Derivation	Retention (%)	
		Absence of stabilizer	Presence of stabilizer
AST	Human liver gene recombinant	41	90
AST	Pig cardiac muscle	44	93
AST	Pig cardiac muscle	43	94
ALT	Human liver gene recombinant	20	92
ALT	pig cardiac muscle	21	91
ALT	Pig cardiac muscle	20	90

As is apparent from Table 5, valine and proline exerted the stabilization effect on various AST(s) and ALT(s) derived from different origins.

Industrial Applicability

A method of stabilizing an enzyme and an enzyme composition of the present invention are concerned with a control substance used in medical examination and are capable of sufficiently stabilizing AST or ALT contained in serum, a buffer or a medium such as soluble protein solution. Thus, the present invention has industrial applicability by providing a method of and a material for obtaining accurate and stable examined data in the field of the medical clinical examination.

CLAIMS

1. A method of stabilizing an enzyme, which comprises incorporating, as a stabilizer for stabilizing at least one enzyme selected from the group consisting of aspartate aminotransferase and alanine aminotransferase, at least one amino acid selected from valine and proline in at least one medium selected from the group consisting of serum and buffer, with a proviso that said proline is incorporated in the amount which exceeds 100 mmol/L when using said proline alone as said stabilizer in case an object to be stabilized is alanine aminotransferase and aspartate aminotransferase is not contained.

2. The method of stabilizing an enzyme according to claim 1, wherein the content is controlled within a range from 0.5 to 100 mmol/L when using valine alone among valine and proline.

3. The method of stabilizing an enzyme according to claim 1, wherein the valine content is controlled within a range from 5 to 20 mmol/L and the proline content is controlled within a range from 10 to 500 mmol/L when using valine and proline in combination.

4. The method of stabilizing an enzyme according to claim 1, wherein the content is controlled within a range from 0.5 to 500 mmol/L when using proline alone among valine and proline for stabilization of aspartate aminotransferase.

5. The method of stabilizing an enzyme according to claim 1, wherein said serum or buffer is a buffer containing a soluble protein.

6. The method of stabilizing an enzyme according to claim 5, wherein said soluble protein is at least one soluble protein selected from the group consisting of albumin and gelatin.

7. The method of stabilizing an enzyme according to claim 6, wherein the concentration of said albumin is within a range from 0.5 to 15W/V%.

8. The method of stabilizing an enzyme according to claim 6, wherein the concentration of said gelatin is within a range from 0.5 to 15W/V%.

9. An enzyme composition comprising at least one medium selected from the group consisting of serum and buffer, at least one enzyme selected from the group consisting of aspartate aminotransferase and alanine aminotransferase and a stabilizer for stabilizing said enzyme, which is made of at least one amino acid selected from valine and proline, said enzyme and said stabilizer being incorporated in said medium, with a proviso that said proline is incorporated in the amount which exceeds 100 mmol/L when using said proline alone as said stabilizer in case an object to be stabilized is alanine aminotransferase and aspartate aminotransferase is not contained.

ABSTRACT

Disclosed is a method for the purpose of performing a medical examination using aspartate aminotransferase or alanine aminotransferase accurately and stably by stabilizing aspartate aminotransferase or alanine aminotransferase in a medium, and valine or proline, as a stabilizer for stabilizing aspartate aminotransferase or alanine aminotransferase, is incorporated in the medium such as serum and buffer.

Docket No.

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on 23 April 1999 as United States Application No. ~~XXXX~~ or PCT International Application Number PCT/JP99/02205 and was amended on _____

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)
Patent Appln.

Priority Not Claimed

10-131159
(Number)

Japan
(Country)

24 April 1998
(Day/Month/Year Filed)

☒ ☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

_____	_____
(Application Serial No.)	(Filing Date)
_____	_____
(Application Serial No.)	(Filing Date)
_____	_____
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

_____	_____	_____
(Application Serial No.)	(Filing Date)	(Status)
		(patented, pending, abandoned)
_____	_____	_____
(Application Serial No.)	(Filing Date)	(Status)
		(patented, pending, abandoned)
_____	_____	_____
(Application Serial No.)	(Filing Date)	(Status)
		(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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